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β_3 INTEGRIN DERIVED PEPTIDE 217-230 INHIBITS FIBRINOGEN BINDING AND PLATELET AGGREGATION: SIGNIFICANCE OF RGD SEQUENCES AND FIBRINOGEN Aα-CHAIN

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Summary. β_3 integrin derived peptides 217-230 (DAPEGGFDAIMQAT) and 217-231(Y) (DAPEGGFDAIMQATVY) at 100 μM inhibited ^{125}I-fibrinogen binding to ADP-stimulated platelets and platelet aggregation. Peptide 217-231(Y) (100 μM) significantly inhibited the binding of ^{125}I-albolabrin (a disintegrin with a single RGD sequence) to ADP- and thrombin-activated platelets while it had only a slight effect on albolabrin binding to resting platelets. The ^{125}I-β_3 217-231(Y) cross-linked selectively to the fibrinogen Aα-chain. The interaction of the RGD sequence in the Aα-chain of fibrinogen with β_3 217-231 sequence appears to play a significant role in the events leading to platelet aggregation.

An α_{IIb}/β_3 integrin (glycoprotein IIb/IIIa) is the most abundant platelet membrane component. Upon stimulation by agonists, such as ADP, thrombin, and collagen, α_{IIb}/β_3 binds adhesive ligands, such as fibrinogen, fibronectin, von Willebrand Factor, vitronectin, and thrombospondin (1).

It has been proposed that platelet recognition sites on fibrinogen are located in the C-terminal end of the γ-chain (amino acids 400-411) and in the N-terminal (amino acids 95-98) and the C-terminal domains (amino acids 572-575) of the Aα-chain of fibrinogen (2,3).

There is evidence that multiple fibrinogen binding sites are located on the β_3 subunit of the α_{IIb}/β_3 complex. It has been suggested that the adhesive binding domain of β_3 is between amino

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acid residues 130-347 (4) or 101-422 (5). Using chemical cross-linking, d'Souza et al. (6) demonstrated that β_3 domain, spanning from amino acid 107-171, is involved in binding short RGD peptides. Andrieux et al. (7) suggested that β_3 domain 108-128 is cryptic on resting platelets. Charo et al.(8) demonstrated that peptides β_3 204-222, β_3 211-229, and β_3 211-222 blocked binding of fibrinogen to purified receptor and concluded that β_3 domain 211-222 is critically involved in adhesive protein binding. Our previous work in a solid phase system (9) demonstrated that β_3 derived peptide 217-231(Y) binds to fibrinogen and to von Willebrand Factor, and it is displaced from its binding site by the purified α_{IIb}/β_3 complex. Moreover, an analogous peptide in which $proline_{219}$ was substituted with alanine did not bind to fibrinogen.

We report here the inhibitory effect of β_3 217-230 peptide on ADP-induced platelet aggregation, on ^{125}I-fibrinogen binding to ADP-stimulated platelets, and on ^{125}I-albolabrin binding to ADP- and thrombin-stimulated platelets. Albolabrin belongs to the disintegrin family of low molecular weight, cysteine rich peptides which occur in viper venoms and are potent inhibitors of platelet aggregation and cell adhesion (10-12). Since the RGD sequence represents a single cell recognition epitope in disintegrins, albolabrin has been used as a tool to search for supportive evidence that the interaction of β_3 217-230 sequence with fibrinogen occurs through an RGD dependent mechanism. Moreover, our study suggests that β_3 217-230 peptide blocks platelet aggregation by interacting with the fibrinogen Aα-chain.

MATERIALS AND METHODS

Fibrinogen: Human fibrinogen (Kabi, Stockholm, Sweden) was utilized in platelet aggregation and binding studies. Highly purified fibrinogen (Band I) prepared according to Pandya and Budzynski (13) was used for cross-linking studies.

Peptide synthesis and purification: Peptides (1700 Da), containing the β_3 217-230 sequence, were synthesized at the Wistar Institute (Philadelphia,PA) or at MPS (San Diego, CA) by the solid phase method: peptide A, DAPEGGFDAIMQAT; peptide A(Y), DAPEGGFDAIMQATVY; peptide B, DAAQGGFDAIMQAT; and peptide C, DAAEGGFDAIMQAT. For labeling purposes, tyrosine had been added to the C-terminal end of peptide A; P_{219} and E_{220} were substituted with A_{219} and Q_{220} in peptide B; A_{219} replaced P_{219} in peptide C. Following purification by high pressure liquid chromatography (Waters, Milford, MA) on reverse phase C-18 column (Vydac, Hesperia, CA), the purity of peptides was confirmed by amino acid analysis (Wistar Institute) and mass spectral analysis (MPS).

Disintegrins: Albolabrin (7500 Da) was purified from snake venom of Trimeresurus albolabris (Latoxan, Rosans, France) by the

modified methods of Williams (14). Briefly, albolabrin was applied to a Mono Q ion exchange column (Pharmacia, Uppsala, Sweden) in 0.05 M Tris buffer and eluted with NaCl gradient. Fractions containing albolabrin (0.7 M NaCl), as detected by rabbit anti-albolabrin antibody in a solid phase system, were further purified by reverse phase HPLC (C-18 column). The activity was measured in platelet aggregation inhibition assay.

Platelet aggregation studies: Human platelets were prepared according to a modified method of Mustard et al. (15). Aggregation studies, with 60 μM ADP, were performed in a Payton aggregometer (Scarborough, Ontario, Canada).

^{125}I-Labeling of proteins and peptides: Fibrinogen, albolabrin and peptide A(Y) were labeled using Iodobeads (Pierce, Rockford, IL), and ^{125}I-Na (Amersham, Arlington Heights, IL) as described previously (9). Specific radioactivity of radiolabeled fibrinogen and albolabrin was approximately 45.0 - 55.0 kBq per μg.

Binding of ^{125}I-fibrinogen and ^{125}I-albolabrin to platelets: This was studied as described by Huang et al. (12).

Cross-linking of ^{125}I-peptide A(Y) to fibrinogen: Band I fibrinogen (4.5 mg/ml) in 50 mM HEPES buffer, pH 9.2, was incubated with ^{125}I-peptide A(Y) at room temperature for 30 min. The radiolabeled peptide was cross-linked to fibrinogen using a homobifunctional cross-linking reagent, disuccinimidyl suberate (DSS, Pierce, Rockford, IL) at 22° C. After 20 min, the reaction was stopped by addition of 2 M glycine and 2% SDS. Analysis was carried out by autoradiography of a Coomassie Blue-stained gel (SDS-PAGE) in the non-reduced (7%) and reduced (15%, 0.1% mercaptoethanol) systems.

RESULTS AND DISCUSSION

We examined the effects of synthetic peptide β_3 217-230 and three analogs on platelet aggregation in the presence of ADP. Peptides A(Y) and A, at 100 μM concentration, caused more than 70% inhibition of platelet aggregation; however, RGES, peptides B, and C had no effect (Fig. 1a). Additionally, peptides A(Y), A, and RGDS, at 10 μM concentration, produced about 50% inhibition of platelet aggregation, whereas RGES, peptides B and C had no effect (data not shown). Fig.1b illustrates a dose-dependent, saturable binding of ^{125}I-fibrinogen to washed human platelets stimulated by 60 μM ADP. Preincubation of ^{125}I-fibrinogen with 100 μM peptide A(Y) significantly decreased this binding. Binding of ^{125}I-fibrinogen to resting platelets was the same in the absence or presence of peptide A(Y), demonstrating that peptide A(Y) did not affect non-specific fibrinogen binding. Further studies confirmed that peptides A and A(Y) inhibited fibrinogen binding in a similar dose-dependent manner, whereas peptides B and C had no effect (data not shown).

To evaluate the interaction of ^{125}I-peptide A(Y) with fibrinogen, we cross-linked ^{125}I-peptide A(Y) to fibrinogen using disuccinimidyl suberate and analyzed the Coomassie Blue-stained

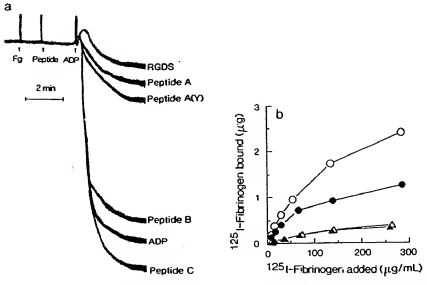

Fig.1. PLATELET AGGREGATION AND FIBRINOGEN BINDING TO PLATELETS IN THE PRESENCE OF β_3-INTEGRIN DERIVED PEPTIDES.

a) The β_3 integrin derived peptides and RGDS, at 100 μM, were added to washed platelets (5 x 10^8/mL) in buffer containing 2 mM $CaCl_2$ and fibrinogen (0.6 mM). Aggregation was initiated with ADP (60 μM). Tracing is a representative one of 15 similar experiments.

b) Binding of ^{125}I-fibrinogen (20-300 μg/mL) to washed platelets (3 x 10^8/ml) stimulated by 60 μM ADP in the absence (○) or presence (●) of 100 μM peptide A(Y). Nonspecific fibrinogen binding to unstimulated platelets was measured in the absence (△) or presence (▲) of the peptide. Data represent mean values from 3 identical experiments.

gels by autoradiography. As presented in Fig. 2, the non-reduced, 7% gel (lane A) and autoradiograph (lane B) shows that fibrinogen (MW=340,000 Da) intra-chain cross-linking was minimal. In the reduced gel (lane C), the arrows indicate the individual fibrinogen chains (Aα=68,000; Bβ=58,000; γ^4=47,000 Da) while from its autoradiograph (lane D), it can be seen that the radiolabeled peptide cross-linked selectively to the Aα-chain of fibrinogen.

^{125}I-albolabrin binds to resting and ADP- or thrombin-activated platelets in a saturable manner. Scatchard analysis (Fig.3a) shows that albolabrin binds to a similar number of sites on resting, ADP- and thrombin-activated platelets. However, ADP and thrombin increased the binding affinity of albolabrin 4-fold and 14-fold, respectively. Peptide A(Y) significantly inhibited ^{125}I-albolabrin binding (low concentrations) to thrombin-activated platelets (50%) and to ADP-activated platelets (40%): however, it had only slight effect (13%) on binding of this disintegrin to resting platelets (Fig. 3b). An increase of the inhibitory effect of peptide A(Y) on

Fig.2 CROSS-LINKING OF PEPTIDE A(Y) TO FIBRINOGEN.
Cross-linking of peptide A(Y) to band I fibrinogen was accomplished using DSS. The molar ratio of peptide to fibrinogen was 7:1 and of DSS to fibrinogen, 54:1. Analysis of a 7%, non-reduced (lane A) and a 15%, reduced (lane C) SDS-PAGE by autoradiography (lanes B and D, respectively) indicates that the ^{125}I-peptide A(Y) cross-linked to the Aα-chain of fibrinogen.

albolabrin binding to thrombin-activated platelets as compared to resting platelets was highly significant, since binding affinity of this disintegrin increased several-fold following platelet activation (Fig. 3a). EDTA completely inhibited binding of

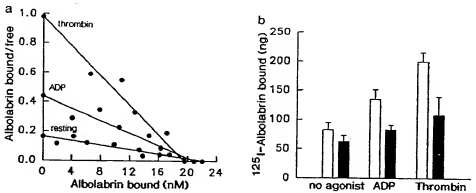

Fig.3. BINDING OF ^{125}I-ALBOLABRIN TO RESTING OR TO STIMULATED PLATELETS.
a) Various concentrations of ^{125}I-albolabrin were added to platelets ($3x10^8$/ml) prior to incubation with ADP (60μM), thrombin (0.1U/ml) or buffer. The number of albolabrin binding sites on resting, ADP and thrombin activated platelets were 90,200 (Kd 258 nM), 80,100 (Kd 80 nM), and 100,200 (Kd 46 nM), respectively. Data are representative of three similar experiments.
b) ^{125}I-albolabrin (1 μg/ml) was incubated without (opened bars) or with (closed bars) 100 μM of peptide A(Y) and added to platelets (3×10^8/ml). After 1 min platelets were stimulated with thrombin (0.1 U/mL) or ADP (60 μM). Control represents binding of albolabrin to unstimulated platelets. The ordinate shows albolabrin binding in ng per 3×10^8 platelets. Data represent mean values from three experiments.

radiolabeled albolabrin to platelets regardless of the activation state (data not shown).

The current study shows that peptides A and A(Y), at relatively low concentrations, inhibited ADP-induced platelet aggregation and fibrinogen and albolabrin binding to stimulated platelets, whereas peptides with $proline_{219}$ substituted with alanine did not have such an effect. It appears that the interaction of the 217-230 region of β_3 with fibrinogen molecule is RGD dependent since peptides A and A(Y) inhibited binding of albolabrin to platelets and since albolabrin belongs to a family of disintegrins containing RGD as a single cell recognition site (10-12). Accordingly, we also demonstrated that binding of highly purified peptide A(Y) to immobilized fibrinogen was inhibited by albolabrin and RGDS but not by RGES (unpublished observation). Increase of the inhibitory activity of the peptide on the binding of ^{125}I-albolabrin to stimulated platelets suggests that the β_3 region encompassing peptide A remains cryptic on resting platelets and is exposed following platelet activation. Although the significance of the C-terminal end of the fibrinogen γ'-chain is well established (2,3,16,17), the role of the Aα-chain in fibrinogen binding and platelet aggregation is more controversial (16-21). According to Hawiger et al.(21) isolated Aα-chain and γ'-chain, in contrast to Bß-chain, support platelet aggregation. Most recently, Farrell et al.(16) expressed human recombinant fibrinogen containing variant of the γ'-chain and determined that this mutant did not support platelet aggregation, although two RGD sequences in the Aα-chain were not substituted. In contrast, substitution of aspartic acid with glutamic acid in two RGD sequences of the Aα-chain did not have any significant effect. Our experiments, employing a different approach, suggest that fibrinogen Aα-chain is important for platelet aggregation. It appears that selective interaction of β_3 integrin-derived peptide 217-230 with fibrinogen Aα-chain resulted in the inhibition of fibrinogen binding to platelets and in the inhibition of ADP-induced platelet aggregation. Experiments with albolabrin suggested that this peptide recognizes RGD sequences. Therefore, it is conceivable that the location of cross-linked peptide can be in the vicinity of RGDF (amino acids 95-98) or RGDS (amino acids 572-575) and that the epitope recognizing Aα-chain is located on the β_3 within amino acids 217-230. On the other hand, the epitope recognizing C-terminal peptide of the fibrinogen γ'-chain was previously located on the surface of the heavy chain of α_{IIb} within amino acids 294-314 (22).

In conclusion, our observation is compatible with the hypothesis that there are multiple fibrinogen binding sites on the α_{IIb}/β_3 receptor and multiple platelet recognition sites on the fibrinogen molecule.

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